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RESTORATION OF THE COELOMOCYTE SYSTEM IN THE FED AND FOOD-DEPRIVED EARTHWORMS *ALLOLOBOPHORA CHLOROTICA AND DENDROBAENA VENETA*

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We have previously shown that the earthworms *Dendrobaena veneta* survive a loss of coelomocytes caused by the electric shock (1 min, 4.5V) and are able to restore their immune system at a temperature-dependent rate, being faster at 20°C than at 10°C. The aim of the present study (conducted at 16°C) was to compare the restoration of immunocompetent cells in two ecologically contrasting lumbricid species, i.e. endogeic *Allolobophora chlorotica* and epigeic *Dendrobaena veneta*, being either fed ad libitum nettle and dandelion leaves (F) or food-deprived (U – unfed). In both species, food deprivation inhibited body weight gain and almost completely inhibited cocoon production. The latter was also partly inhibited during restoration of the immune system (R groups). In comparison with earthworms possessing the intact immune system (C – control groups), the number of coelomocytes was still (although statistically insignificantly) diminished 6-7 weeks after experimental expulsion; among them the amoebocytes were fully restored while the number of eleocytes was still significantly reduced. In both species, the riboflavin content of coelomocyte lysates was diminished during restoration of coelomocyte systems. The amount of riboflavin per eleocyte showed a clear tendency to rise in recovering eleocytes, as compared with that in the intact worms.

Key words: *Allolobophora chlorotica; Dendrobaena veneta;* amoebocytes; eleocytes; riboflavin;

INTRODUCTION

The earthworm immune system is very efficient (BILEJ et al. 2011). Their immunocompetent cells, the coelomocytes, contain amoebocytes, being classical immunocytes (according to OTTAVIANI'S nomenclature, 2011), plus a species-specific portion of chloragogen tissue-derived free chloragocytes (eleocytes) (PLYTYCZ et al., 2009). The eleocytes (detached chloragocytes), but not amoebocytes, exhibit autofluorescence (CHOLEWA et al., 2006) restricted to chloragosomal vesicles, as

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evidenced by confocal microscope (Plytycz et al., 2007). Autofluorescent self-marking predisposed these cells for analysis by flow cytometry (e.g. Cholewa et al., 2006; Plytycz et al., 2011b). Studies by spectrofluorimetry revealed that riboflavin (Koziol et al., 2006) stored in chloragosomes of chloragocytes (in the preparation) and chloragocyte-derived eleocytes is one of fluorophores responsible for their autofluorescence (Cygal et al., 2007). The percentage of autofluorescent eleocytes among coelomocytes, and the amount of riboflavin stored in eleocytes are species-specific (Plytycz et al., 2006) and changes in response to various edaphic factors (Plytycz et al., 2011a), including metal soil contamination (e.g. Homa et al., 2010; Kwadrans et al., 2008; Piotrowska et al., 2010; Podolak et al., 2011). Lumbricid worms can expel coelomocytes through dorsal pores when irritated under natural conditions (e.g. by predators) or under strictly controlled experimental conditions by mild electric current (Roch, 1979), ultrasounds (Hendawi et al., 2004) or 5% ethanol (Cooper at al., 1995). Such a treatment does not affect worm viability and their immune system gradually recovers. We have previously shown that recovery of the initial coelomocyte number by *Dendrobaena veneta* subjected to the electric shock is a long-lasting temperature-dependent process (Olchawa et al., 2003). The aim of the present work was to compare the coelomocyte systems (i.e. the number of amoebocytes and eleocytes, and the riboflavin content of coelomocyte lysates) several weeks after experimental extrusion of coelomic fluid by epigeic *Dendrobaena veneta* and endogeic *Allolobophora chlorotica* earthworms, and to determine the effects of food deprivation on the recovery of the coelomocyte system.

MATERIALS AND METHODS

Earthworms

Adult specimens of *Allolobophora chlorotica* were collected from a relatively metal-free site in Krakow; *Dendrobaena veneta* were purchased from a commercial supplier (EKARGO Slupsk). The experiments were conducted in the laboratory of the Institute of Zoology, Jagiellonian University in Krakow, under controlled conditions (16 ±1°C; 12:12 LD). The worms were kept in plastic boxes with perforated lids and the moisture content was checked weekly.

Scheme of the experiments

For the experiments, groups of 32 worms belonging to each species were transferred to fresh samples of commercial soil, 8 animals per box, and were kept for 6 (*A. chlorotica*) or 7 weeks (*D. veneta*). Within each species, half of the worms possessed intact immune systems (control, C groups), and half of them were deprived of coelomocytes on day 0 by electric shock (see below), thus they were undergoing the process of restoration of coelomocytes (restoration, R groups). Within C and R groups, the animals were either fed ad libitum a mixed diet comprised of dried/boiled nettle (*Urtica dioica*) and dandelion (*Taraxacum officinale*) leaves (F groups) or deprived of food supply (unfed – U groups). Thus, within each species four experimental groups were formed: CF, RF, CU, RU in four boxes, 8 worms per box. At the end of the experiments the worms were weighed, coelomocytes of all the animals were extruded and analysed, and cocoons in each box were counted.

Coelomocyte extrusion

Earthworms were stimulated for 1 minute by electric current (4.5V) to expel coelomic fluid with suspended coelomocytes through the dorsal pores, according to the procedure described previously (Kwadrans et al. 2007). Briefly, the weighed earthworms were placed individually in Petri dishes containing 3 mL of extrusion fluid (phosphate-buffered saline, PBS, supplemented with 2.5 g/L ethylenediamine tetra-acetic acid, EDTA to avoid cell aggregation). Extruded coelomocytes were counted in a haemocytometer; 1 mL suspensions were used for spectrofluorimetry and the remaining sample from each worm was fixed in 2% formalin for flow cytometry.

Flow cytometric measurement and analysis

Samples of coelomocytes were analysed with a FACScalibur flow cytometer (BD Biosciences). During analytical experiments, 10000 threshol-

ded events per worm sample were collected and analysed on the basis of their forward scatter (FS) (for cell size) and sideward scatter (SS) (cell complexity). Fluorescence FL1 (emission 530 nm; excitation 488 nm) was recorded. The resulting files were analysed using WinMDI 2.9 software (Joe Trotter, http://facs.scripps.edu), by producing dot plots and histograms of FL-1H autofluorescence.

Spectrofluorimetric measurements and analysis

The spectrofluorimetric measurements were performed on coelomocyte suspension lysates (lysed with 2% Triton; Sigma-Aldrich) using Perkin-Elmer Spectrofluorimeter LS50B. Excitation spectra were recorded between 300-520 nm (excitation at 525 nm), while emission spectra were recorded between 380-700 nm (excitation at 370 nm). The spectrofluorimetric signatures of unbound riboflavin were characterized by two maxima (at 370 nm and 450 nm) in the excitation spectrum and a maximum at 525 nm in the emission spectrum. Arbitrary units (AU) of fluorescence were recorded using Microsoft Excel v. 97.

Statistical analysis

The results were expressed as means ± standard errors. Differences between the means were determined by Student's t-test (Microsoft Excel v. 97), with the level of significance established at $p<0.05$.

RESULTS

Cocoon production (Fig. 1)

Weekly cocoon production by worms fed ad libitum a vegetarian diet (FC groups) was much higher in the intact *D. veneta* than *A. chlorotica*. In both species the number of cocoons was reduced in the fed animals restoring the coelomocyte systems (FR groups). The lowest number of cocoons was observed in food-deprived worms, both in those with the intact coelomocytes (UC) and those restoring their coelomocyte systems (UR) (Fig. 1).

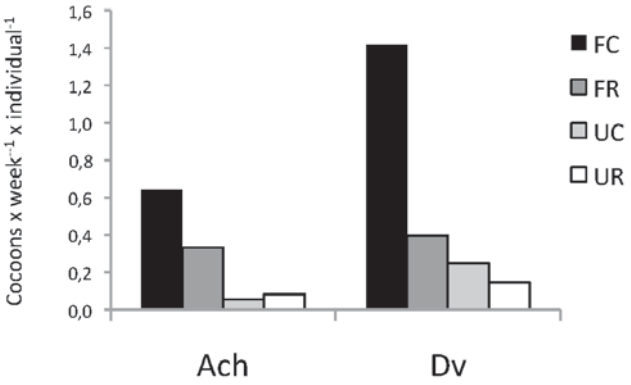

Fig. 1. Cocoon production by *Allolobophora chlorotica* (Ach) and *Dendrobaena veneta* (Dv) fed ad libitum (F) nettle and dandelion leaves or food-deprived (U, unfed), which were for 6 and 7 weeks, respectively, either kept intact (C) or underwent the process of restoring (R) the extruded coelomocytes. FC – fed control; FR – fed, restoring coelomocytes; UC – unfed control; UR – unfed, restoring coelomocytes. Y axis – number of cocoons per week per worm. Average number per week per earthworm, for 8 worms per group.

Body weights (Fig. 2)

Within each species, initial body weights of worms were similar. During 6-week (*A. chlorotica)* and 7-week (*D. veneta)* experiments the worm body weights were significantly increased in the animals

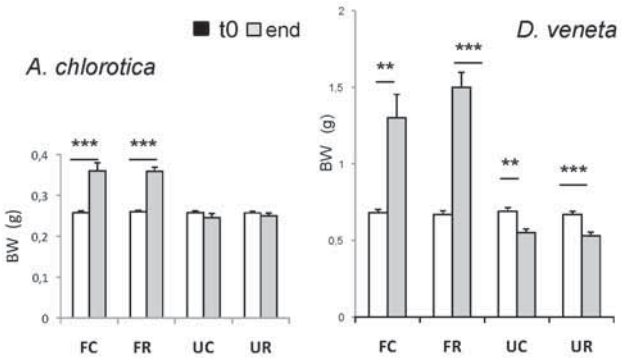

Fig. 2. Body weights (BW) of adult *Allolobophora chlorotica* (Ach) and *Dendrobaena veneta* (Dv) earthworms at the start (open bars, time 0) and at the end (solid bars) of the experiments. The worms were (for 6 and 7 weeks, respectively) either kept intact (C, control) or underwent the process of restoring (R) the extruded coelomocytes, being either fed ad libitum nettle and dandelion leaves (F) or food-deprived (U – unfed): FC – fed control; FR – fed, restoring coelomocytes; UC – unfed control; UR – unfed, restoring coelomocytes. Means±SE, n= 8 worms per group. Differences between initial and final body weights within a group, which are statistically significant at $^{**}p<0.01$ or $^{***}p<0.001$, are denoted by asterisks.

fed ad libitum nettle and dandelion leaves, both in the worms with intact coelomocytes (FC groups) and those restoring the extruded coelomocytes (FR groups). Body weight gain was inhibited in food-deprived *A. chlorotica*, in the worms belonging to both the unfed UC and UR groups, while the unfed *D. veneta*, the worms belonging to both UC and UR groups, showed even a reduction in body weights as compared with the values at the start of the experiments, which was statistically significant (Fig. 2). Within each species, final body weights were similar in the fed animals (FC and FR groups), being significantly higher than those in the unfed worms (UC and UR, being similar to each other) (not shown on the graph for clarity reasons).

Coelomocyte composition (Fig. 3)

In both fed (F) and unfed (U) groups of *A. chlorotica* and *D. veneta*, flow cytometry revealed that the percentage of eleocytes was always lower in the worms recovering their coelomocytes depleted on day 0 by the electric shock (R) than in their control (C) counterparts. The differences between control (C) and recovery (R) groups were statistically significant in the fed *A. chlorotica*, and in both fed and unfed *D. veneta*.

Within each species, the percentage of eleocytes was similar in the control worms, either fed or un-

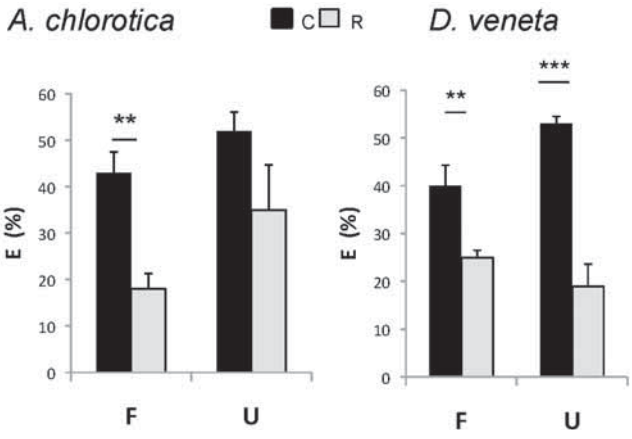

Fig. 3. Percentage of autofluorescent eleocytes (E) of adult *Allolobophora chlorotica* (Ach) and *Dendrobaena veneta* (Dv) earthworms at the end of the experiments. The worms were (for 6 and 7 weeks, respectively) either kept intact (C, control) or underwent the process of restoring (R) the extruded coelomocytes, being either fed ad libitum nettle and dandelion leaves (F) or food-deprived (U – unfed): FC – fed control; FR – fed, restoring coelomocytes; UC – unfed control; UR – unfed, restoring coelomocytes. Means±SE, n= 8 worms per group. Differences between groups of worms recovering coelomocytes and their respective controls, which are statistically significant at **p<0.01 or ***p<0.001, are denoted by asterisks.

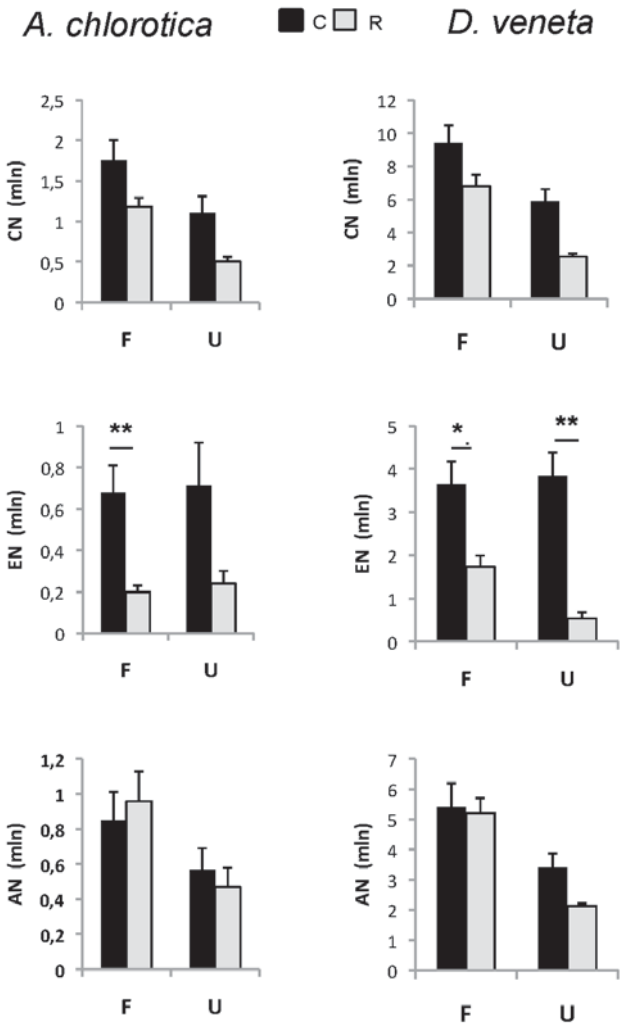

Fig. 4. The number of coelomocytes (CN), eleocytes (EN) and amoebocytes (AN) of adult *Allolobophora chlorotica* (Ach) and *Dendrobaena veneta* (Dv) earthworms at the end of the experiments. The worms were (for 6 and 7 weeks, respectively) either kept intact (C, control) or underwent the process of restoring (R) the extruded coelomocytes, being either fed ad libitum nettle and dandelion leaves (F) or food-deprived (U – unfed): FC – fed control; FR – fed, restoring coelomocytes; UC – unfed control; UR – unfed, restoring coelomocytes. Means±SE, n= 8 worms per group. Differences between groups of worms recovering coelomocytes and their respective controls, which are statistically significant at *p<0.05 or **p<0.01, are denoted by asterisks.

fed (FC and UC groups). Within each species, the differences between FR and UR groups recovering their coelomocyte systems were not statistically significant.

Coelomocyte number (Fig. 4)

Within both F and U groups of both the species, the number of coelomocytes (CN) counted using a haemocytometer was much lower in the recovery groups (R) than in their respective controls (C), but these differences were statistically insignificant (Fig. 4). The percentage of eleocytes established by flow cytometry (see Fig. 3) made it possible to calculate the number of eleocytes (EN) in each individual, and then the number of amoebocytes (AN) according to the formula: AN = CN – EN.

The number of eleocytes was always much lower in the recovering (R) groups of both species than in their respective controls (C), which was statistically significant in all the groups except the unfed groups (U) of *A. chlorotica*. In both the species, at the end of experiments the number of amoebocytes in worms recovering their immunocompetent cells was similar to that in worms with intact immune systems (Fig. 4).

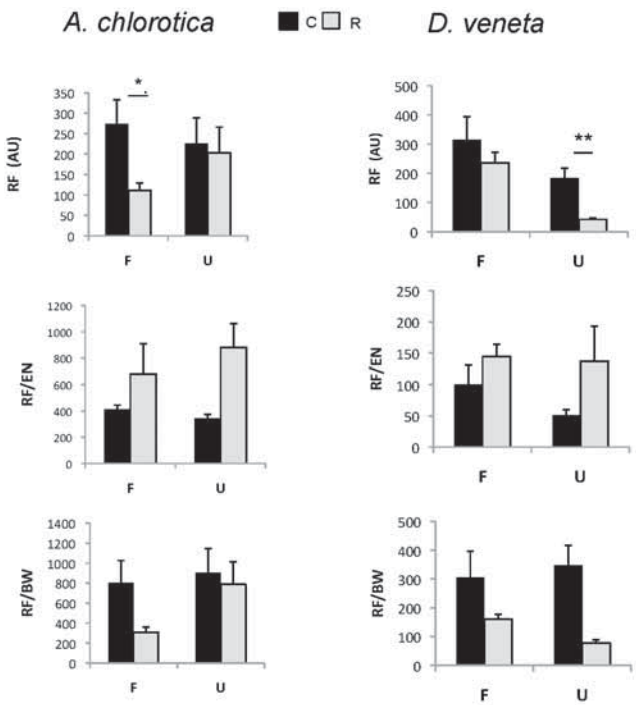

Fig. 5. The riboflavin (RF) content of coelomocyte lysates of adult *Allolobophora chlorotica* (Ach) and *Dendrobaena veneta* (Dv) earthworms at the end of the experiments. The worms were (for 6 and 7 weeks, respectively) either kept intact (C, control) or underwent the process of restoring (R) the extruded coelomocytes, being either fed ad libitum nettle and dandelion leaves (F) or food-deprived (U – unfed): FC – fed control; FR – fed, restoring coelomocytes; UC – unfed control; UR – unfed, restoring coelomocytes. Means±SE, n= 8 worms per group. Differences between groups of worms recovering coelomocytes and their respective controls, which are statistically significant at *p<0.05 or **p<0.01, are denoted by asterisks.

Riboflavin content (Fig. 5)

The riboflavin (RF) content of coelomocyte lysates, in arbitrary units, was statistically significantly reduced in the recovery group of fed (FR) *A. chlorotica* and in the unfed (UR) *D. veneta* versus their respective controls (CR). In both the species, the amount of riboflavin per eleocyte (RF/EN) shows a clear (but statistically insignificant) tendency to rise in recovering eleocytes (FR and UR groups), as compared with that in the intact worms (FC and UC groups). A tendency towards a reduction in the riboflavin content per body weight (RF/BW) was observed in all but the unfed *A. chlorotica* group.

DISCUSSION

It was previously shown that the earthworm *Dendrobaena veneta* irritated by electric current (1 min, 4.5V) extruded from the coelomic cavity up to 95% of coelomocytes and such a loss of immunocompetent cells did not affect the animal viability. During 24 hours after extrusion about 50% of the initial number of coelomocytes were restored. This number did not change during the 6-week experimental period in worms kept at 10°C, while it was restored to the control level during 3-4 weeks in animals kept at 20°C (Olchawa et al., 2003). The present studies were performed at 16°C as such a temperature was optimal for both the investigated species, endogeic *Allolobophora chlorotica* and epigeic *Dendrobaena veneta*, as indicated by efficient cocoon production by worms fed ad libitum a vegetarian diet. Cocoon production was drastically inhibited in animals restoring their immune system and even more in those deprived of food. It sugge-

sts that restoration of coelomocytes is an energy demanding process, competing with reproduction. Further experiments with statistical analysis are necessary to verify such an assumption.

Food deprivation caused inhibition of body weight gain in endogeic *A. chlorotica* and a reduction in the initial body weight in epigeic *D. veneta*. Such body weight gains or losses were similar in the intact and coelomocyte-deprived individuals of both the species. It indicates that restoration of the coelomocyte system does not affect body weight fluctuations which are dependent mainly on a proper diet. To sum up, the studies on restoration of the earthworm immune system may be a new attractive model for studies on energetic costs of immunity from an evolutionary perspective as this issue is still a matter of controversy (e.g. see Boughton et al., 2007; Segerstrom, 2007; Allen and Little, 2011).

In the present studies, the total number of coelomocytes in *A. chlorotica* and *D. veneta* was still diminished (although statistically insignificantly) 6 and 7 weeks after experimental expulsion in comparison with that in the control intact counterparts of these species. However, the number of amoebocytes already reached the species-specific control levels, and diminution was caused exclusively by lack of restoration of the number of eleocytes. The percentage (established by flow cytometry) and number of eleocytes were always lower in worms subjected to experimental expulsion of coelomic fluid. We assume that some uncontrolled stimulations of earthworms during transportation or laboratory routines may induce unintentional extrusion of coelomocytes leading to a relatively fast recovery of amoebocytes but much slower reconstruction of the cohort of eleocytes, being the detached chloragocytes. This may be the reason for high standard deviations and standard errors in the experimental groups of several studies.

In the present experiments, the riboflavin content recalculated per body weight was similar in the fed on nettle and dandelion leaves and food-deprived intact *A. chlorotica* and *D. veneta* worms. In the previous experiments, *Eisenia andrei*, *A. chlorotica* and *D. veneta* worms were fed a mixed diet comprised of flour, boiled/dried/powdered tea leaves and powdered mouse feed (4:1:1) or deprived of food supply for 7 weeks. The results indicated that 7-week food deprivation did not cause riboflavin depletion (when calculated per body weight) in the intact individuals of these 3 worm species kept in unpolluted reference soil. Therefore the results of both the present and previous experiments suggest that bacteria or fungi inhabiting the surrounding soil or worm intestines (e.g. Thakuria et al., 2008) and tissues (e.g. Lund et al., 2010) may be the main source of riboflavin stored in chloragocomes of chloragocytes and chloragocyte-derived eleocytes of the investigated species.

In the present experiments, the riboflavin content of coelomocyte lysates, both the total content and the content recalculated per body weight, is slightly or significantly diminished during restoration of coelomocyte systems of both the investigated species. Long-term depletion of riboflavin may have deleterious effects on earthworm immunity as riboflavin (vitamin B2) helps maintain the proper balance between the worm immune system and microbial invaders inhabiting the animal coelomic cavity. It is well established that riboflavin affects the proper functioning of innate immunity of both animals (e.g. Powers, 2003; Verdrengh and Tarkowski, 2005) and plants (Dong and Beer, 2000), and plays a role in bacterial quorum sensing (Rajamani et al. 2008).

The present investigations revealed that the amount of riboflavin per eleocyte is higher in freshly restored eleocytes than in those from intact worms. We may assume that chloragosomal membranes are more stable in young eleocytes (i.e. freshly detached from the chloragogen tissue) than in "old" eleocytes where leakage of riboflavin may occur. This assumption may be an inspiration for further studies on riboflavin metabolism in earthworm chloragocytes/eleocytes.

In conclusion, the results of the present experiments on *A. chlorotica* and *D. veneta* performed at 16°C have shown that: (1) Food deprivation inhibits body weight gain; (2) Both food deprivation and restoration of immune system inhibit cocoon production in the both species; (3) Both in fed ad libitum and food-deprived worms, 6-7-week restoration of the coelomocyte system led to full recovery of the number of amoebocytes, but not eleocytes, and the riboflavin content; (4) In both the species the riboflavin content was higher in young freshly formed eleocytes than in old eleocytes from intact animals; (5) In consequence, unintentional irritation of earthworms may cause extrusion of coelomocyte-containing coelomic fluid, which has long-term effects on eleocytes and riboflavin content and should be considered when interpreting experimental results.

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